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The distribution of *Aspergillus* spp. opportunistic parasites in hives and their pathogenicity to honey bees

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ABSTRACT

Stonebrood is a disease of honey bee larvae caused by fungi from the genus *Aspergillus*. As very few studies have focused on the epidemiological aspects of stonebrood and diseased brood may be rapidly discarded by worker bees, it is possible that a high number of cases go undetected. *Aspergillus* spp. fungi are ubiquitous and associated with disease in many insects, plants, animals and man. They are regarded as opportunistic pathogens that require immunocompromised hosts to establish infection. Microbiological studies have shown high prevalences of *Aspergillus* spp. in apiaries which occur saprophytically on hive substrates. However, the specific conditions required for pathogenicity to develop remain unknown. In this study, an apiary was screened to determine the prevalence and diversity of *Aspergillus* spp. fungi. A series of dose–response tests were then conducted using laboratory reared larvae to determine the pathogenicity and virulence of frequently occurring isolates. The susceptibility of adult worker bees to *Aspergillus flavus* was also tested. Three isolates (*A. flavus*, *Aspergillus nomius* and *Aspergillus phoenicis*) of the ten species identified were pathogenic to honey bee larvae. Moreover, adult honey bees were also confirmed to be highly susceptible to *A. flavus* infection when they ingested conidia. Neither of the two *Aspergillus fumigatus* strains used in dose–response tests induced mortality in larvae and were the least pathogenic of the isolates tested. These results confirm the ubiquity of *Aspergillus* spp. in the apiary environment and highlight their potential to infect both larvae and adult bees.

1. Introduction

The increasing demand upon honey bees for pollination services and their recent unexplained colony losses has lead to a surge of public and scientific interest in honey bee *Apis mellifera* pathology (Evans and Schwarz, 2011; Aizen and Harder, 2009). Honey bees are faced with multiple stressors such as poor nutrition, pesticides and exposure to pathogens, which combined may have a significant impact on colony survival (van Engelsdorp and Meixner, 2010). Thus, identifying the roles of cryptic, as well as more obvious, stressors is important for understanding their possible interactions with other threats. Honey bees are susceptible to high diversity of parasites of which certain newly emerged groups e.g. viruses, *Varroa destructor*, *Nosema ceranae* (Amdam et al., 2004; Fries, 2010; Genersch and Aubert, 2010) have attracted much attention. However, the impacts of other less studied parasites on colony health as well as their interactions with other stressors may also deserve more consideration.

Stonebrood is considered to be a pathogen of low virulence in honey bee colonies yet very little is known regarding the stonebrood and honey bee host–parasite system (Gilliam and Vandenberg, 1988; Bailey, 1968). A number of species from the genus *Aspergillus* are facultative parasites and have been reported as agents of

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stonebrood (Shoreit and Bagy, 1995; Gilliam and Vandenberg, 1988). In colonies showing the symptoms of stonebrood, hard mummified larvae that have been transformed by the fungus are visible in the brood cells (Bailey, 1968). On rarer occasions infections in adult bees have been reported (Batra et al., 1973). *Aspergillus flavus* has been cited as the primary species responsible, with *Aspergillus fumigatus* occurring occasionally (Gilliam and Vandenberg, 1988). *Aspergillus niger* has also been identified as a dominant species in affected colonies, however the contribution that each of these species play in causing overt stonebrood symptoms has not been determined (Shoreit and Bagy, 1995). Though it is generally accepted that pathogenicity occurs only in colonies weakened by other factors, the specific conditions predisposing the onset of disease are not fully understood (Bailey, 1968; Shoreit and Bagy, 1995). In experimental infections, nutritional status has been shown to effect the survival of larvae following *A. fumigatus* exposure (Foley et al., 2012). In addition, *A. flavus* was highly virulent when larvae were maintained at 34 °C and showed decreased virulence following a 24 h cooling period at 27 °C (Vojvodic et al., 2011), so clearly, it is a multifactorial disease with a combination of host and environmental components required for overt disease to occur.

Aspergillus spp. are primarily saprophytic fungi, occurring commonly in soils and on other organic and inorganic substrates. The conidia (asexual spores) are hydrophobic and readily airborne with the capacity to germinate in a wide range of conditions. They are thermotolerant, and capable of growth in temperatures ranging from 12 °C to over 50 °C which has contributed to their success as wide ranging opportunistic pathogens in vertebrates (Bhabhra and Askew, 2005). Aspergillosis is known to occur in all domesticated and in many wild animal species with birds being particularly susceptible to infection (Tell, 2005). In humans, *Aspergillus* causes a range of disease including allergic bronchopulmonary disease (ABPV), aspergilloma and various forms of invasive aspergillosis (Latgé, 1999). Medical developments in immunosuppressive therapies have resulted in a new ecological niche for *Aspergillus* spp. as an important nosocomial disease in people with impaired immune systems causing serious and often fatal mycoses (Latgé, 1999). *Aspergillus fumigatus* is the primary cause of infections among human pathogenic species followed by *A. flavus*, *Aspergillus terreus* and *A. niger* with the severity of the disease highly dependent upon the immune status of the host (Dagenais and Keller, 2009). In agriculture, *A. flavus* is an important opportunistic pathogen of developing seeds such as corn, peanuts and cottonseed, when colonisation by the fungus results in food spoilage due to aflatoxin production (Amaike and Keller, 2011).

Given that *Aspergillus* spp. occur in such close association to bees within the colony and their potential to cause serious disease in organisms with weakened immune systems, this host-parasite relationship deserves more detailed enquiry. In this study, the diversity and relative abundance of isolates of *Aspergillus* spp. isolates in an apiary was examined, and then controlled dosing of individual larvae was carried out to determine the pathogenicity and virulence of the most common species identified from the screening. The susceptibility of adult bees to aspergillosis was also examined by controlled dosing of individual worker bees with an *A. flavus* isolate.

2. Materials and methods

The apiary was located in West Yorkshire, UK, and contained 39 colonies of *Apis mellifera carnica* which were screened for *Aspergillus* spp. fungi in 2010. To assess colony-level prevalence, 10 larvae and 10 live adult worker bees were collected from each colony in April, as well as 22 soil samples from (a) immediately outside hive entrances and (b) 5 m from the hives. To assess within-colony prevalence, 20 nurse bees, which were identified as adult bees that were observed feeding larvae on the brood frames, 20 large larvae (approximately 4–8 days old) and 20 small larvae (approximately 1–3 days old) were collected from three colonies in August. In addition, airborne fungal conidia were sampled in these colonies using sterile agar plates covered in gauze to prevent contact with bees and attached to the top and middle of a central frame in the brood box, while an uncovered sterile agar plate was placed on the floor of each colony to collect fungal conidia carried by the bees. The selective *Aspergillus flavus* and *parasiticus* agar (AFPA), containing 0.1% chloramphenicol and 0.05% streptomycin, was used, which is suitable for the enumeration, growth and identification of *Aspergillus* spp. (Pitt et al., 1983).

Adult worker bees were dissected 24–48 h after collection. The guts (caecum, ventriculus, oesophagus and crop) of adult bees, or whole larvae, were placed in sterile water and homogenised using a sterile pestle. For the colony-level prevalence samples, the guts from all 10 bees were pooled in 500 μl of sterile water and all 10 larvae from each colony similarly pooled in 500 μl of sterile water. Each sample was then divided in half and spread onto two 90 mm AFPA plates. For the within-colony prevalence samples, each of the nurse bee guts or whole larvae were individually homogenised in 200 μl of water and spread on to individual AFPA plates. The plates were incubated at 30 °C and checked daily for the presence of fungal colonies.

Soil samples were collected with a 2.5 cm × 10 cm core from immediately in front of, and 5 m away from, the entrances of every fifth hive ($n = 11$). A 1 g amount of each sample was added to 9 ml of 0.05% Triton-X suspension and vortexed for 1 min to form a suspension. 200 μl of each suspension was then pipetted onto an AFPA media plate which was then incubated at 30 °C and monitored daily for the presence of fungal colonies.

2.1. Fungal identification and quantification

The growth media plates were examined daily for the presence of fungal colonies. As AFPA agar is not strictly selective, *Aspergillus* spp. fungi species were differentiated according to common morphological characteristics as being from section Fumigati, Flavi, Nigri or 'other' depending on the conidial colour. Pure cultures of *Aspergillus* isolates grown on malt extract agar (MEA),

were prepared for molecular identification to confirm morphological findings and identify other species from the genus that were present. To determine an estimate of relative fungal spore intensity per sample in larvae and adult bees, colony forming units (CFU's) were counted on each of the plates for each colony. Assuming that each colony represented a single conidial inoculum from these quantities, the mean number of viable conidia per colony and/or individual was calculated.

To extract the fungal DNA approximately 0.05 g of the fungal conidia were collected from freshly conidiating plates and added to 200 μl 5% Chelex suspension (in 10 mM Tris buffer) and 0.05 g of 0.1 mm Zircona/Silica beads and placed in a QIAGEN Tissue Lyser beadbeater for 4 min at 50 oscillations/s. Samples were then incubated in a 90 °C water bath for 20 min then centrifuged for 30 min at 8 °C. The supernatant was cleaned with OneStep-96 PCR Inhibitor Removal Kit (Zymo Research) prior to PCR. Species identification was carried out by sequencing 547 and 459 bp long fragments from the ribosomal internal transcribed spacer regions 1 and 2 respectively (Henry et al., 2000) and then BLASTn searches carried out. For isolates where BLASTn searches produced *A. niger* spp. matches where it was not possible to distinguish to species level, a 649 bp long fragment of the calmodulin gene was sequenced and a BLASTn search carried out to identify the species (O'Donnell et al., 2000).

2.2. *Aspergillus* virulence in honey bee larvae

Six *Aspergillus* isolates were included in 3 different dose–response experiments on larvae (Experiments 1a, 1b and 1c; see Table 1) where each experiment tested isolates using a different dose range. In each case honey bee larvae were sampled from 4 colonies, asymptomatic of pests and disease and, headed by one year old queens. One to two days old larvae were grafted onto 48 well sterile tissue culture plates. Larvae were grafted using a Swiss grafting tool and placed the same side up onto a 50 μl drop of royal jelly mix at the base off each well that had been warmed to approximately 30 °C. The royal jelly mix consisted of 50% of fresh frozen royal jelly (v/v) (Apitherapy, UK), 6% D-glucose (w/v), 6% D-fructose (w/v) and sterile distilled water and was used to feed larvae according to the Aupinel et al., (2005) protocol. Larvae were kept under conditions of 34 °C at 90% relative humidity in an incubator (Sanyo Environmental Test Chamber).

Conidia suspensions from six different isolates were used to inoculate the honey bee larvae. These were F2 fungi that had been isolated and identified previously in the season. The conidia were harvested from MEA plates and suspended in 1 ml of sterile water and vortexed for 15 s. The conidia were counted with a 0.001 ml Neubauer haemocytometer and the conidia concentrations per ml calculated. This suspension was adjusted to make an approximate 2×10^6 conidia/ml concentration. To test for conidial viability 40 μl of the suspension was added to 200 μl GLEN, a liquid media suitable for the germination of entomopathogenic fungi (Beauvais and Latgé, 1988), and vortexed for 5 s. 15ul of this suspension was micropipetted into six of the 6 mm spots on sterile Teflon coated slides and placed into sterile petri dishes with moist filter paper. One slide was prepared per species. These were incubated for 18–24 h at 30 °C after which time the proportion of germinated conidia per species was calculated. Once the spore suspensions had been adjusted to account for the percentage of non-viable conidia the suspensions were diluted to the desired concentrations. Three separate dose response experiments were carried out each testing a different range of conidia doses (Table 1). The suspensions were diluted to the required concentrations and placed in the incubator at 34 °C for an hour prior to dosing. A standard volume of 5 μl of each conidia concentration was given per dose. The inoculum was pipetted onto the royal jelly food near the mouth parts of the larva to encourage ingestion of the conidia. The larvae were monitored daily for 7 days post-exposure to the fungi under microscope for mortality and signs of stonebrood infection. This was recognised by the presence of hyphae and conidiophores on the cuticle.

2.3. *Aspergillus flavus* virulence against adult honey bees

For the adult dose–response test, frames of sealed brood were obtained from colonies that were asymptomatic of pests and diseases, and kept at 34 °C and 60% RH for the collection of newly emerged workers. The bees were confined in cages in groups of 15 and fed a sterile sucrose suspension (50%, w/w in deionised water). Three-four days following eclosion the bees were starved for 1.5 h and 30 bees per colony, per dose, were each fed 5 μl of sucrose suspension containing either 50, 500, 5000 or 25,000 *A. flavus* conidia or a pure sucrose suspension for the controls. Prior to dosing, bees were anaesthetised by cooling at 4 °C

Table 1
Aspergillus species isolates and conidia concentrations used in the honey bee larvae dose–response experiments.

Experiment 1: Larval dose–response tests			
Experiment	*Aspergillus* species (isolate code)	Isolate source	Treatment doses (conidia/larvae)
1a	*A. fumigatus* (G23)	Adult bee guts	5, 50, 500 and 5000
	A. flavus (G49)	Adult bee guts	
	A. niger (L29)	Larvae	
1b	*A. fumigatus* (N30.04)	Nurse bee gut	50, 500, 5000 and 25,000
	A. phoenicis (N38.02)	Nurse bee gut	
1c	*A. nomius* (G7)	Adult bee guts	2, 5, 50 and 500
	A. flavus (G49)	Adult bee guts	

until they were motionless (approximately 5 min). Once returned to room temperature the suspension was micropipetted onto the mouthparts when the bees started to awaken ensuring complete ingestion of the dose. The bees were then caged according to dose and colony of origin. For 9 days post inoculation the bees were monitored and dead bees were counted and removed daily.

2.4. Statistical analysis

Differences in the numbers of CFU between honey bee life stages (young larvae, old larvae, adults) were analysed in R 2.1.14.1 using a generalised linear model, with a negative binomial distribution and a log link function, with colony included as a factor in the within-colony analyses. The effects of fungal isolates and dose on the survival of larvae and adult bees were analysed with Kaplain–Meier models, using the *survreg* function with death defined as the event and non-constant hazard (Weibull) error distribution. Isolate, dose, colony and interactions between these terms were removed stepwise to obtain the minimum adequate model. For the adult dose–response test data, the *frailty* function was included in order to fit colony as a random term as (Therneau, 2012). Pairwise comparisons of fungal doses were conducted using multiple comparisons from the means of the model using Tukey contrasts in the *multcomp* package.

3. Results

3.1. Colony level prevalence

There was a significant effect of host life stage on the number of fungal CFU's within samples of pooled adult bee guts and larvae ($\chi^2_{1,76} = 88.82$, $P < 0.001$), with CFU's generally found to be higher in larvae than in adults. CFUs also showed a highly aggregated distribution with significant differences in intensities between colonies ($\chi^2_{1,38} = 2142.37$, $P = 0.02$). Cultures from the soil samples showed that there was a significant effect of proximity to colonies on the number of CFU's in soil ($\chi^2_{10,10} = 22.68$, $P = 0.02$), with samples taken from close to the hive entrance having significantly more CFU's than corresponding samples taken 5 m from each hive ($\chi^2_{1,20} = 43.7$, $P < 0.001$).

3.2. Within-colony prevalence

The conidia intensities observed in the individual samples of three colonies differed significantly between life stages ($\chi^2_{2,177} = 252.09$, $P < 0.001$), and this effect of life stage also varied significantly between colonies ($\chi^2_{2,175} = 236.84$, $P < 0.001$). There was a significant interaction between the two factors ($\chi^2_{4,177} = 183.56$, $P < 0.001$). Intensities in small larvae were significantly lower than in large larvae ($P < 0.01$) and there were no differences between adult nurse bee and large larvae. Viable conidia were least prevalent in small larvae in which 41.6% carried viable conidia whereas 88.3% and 81.6% of nurse bees and large larvae respectively carried viable fungal conidia.

The mean airborne viable fungal spore counts of the gauze covered media plates positioned at the top and middle of 3 colonies were 250 (se ± 70.9) and 245 (se ± 179.3) CFU's respectively. No viable fungal colonies appeared on two of the uncovered plates which were positioned at the bottom of each hive with only 10 CFU's counted on the third.

3.3. Aspergillus spp. diversity

The apiary screenings yielded a total of 378 *Aspergillus* isolates from larvae, adult bees, airborne and soil substrates which were identified by gross morphology (Table 2). This may be interpreted as a highly conservative estimate of *Aspergillus* prevalence as it was not possible to identify non-sporulating colonies. Of these 35 *Aspergillus* spp. and 13 non-*Aspergillus* spp. isolates were sequenced and identified using BLASTn with a minimum 97% threshold. Out of the 15 isolates sequenced from section Fumigati all 15 were confirmed to be *A. fumigatus*. Five isolates were sequenced from section Flavi in which *A. flavus* (2/5), *A. oryzae* (2/5) and *Aspergillus nomius* (1/5) were identified. The 18 isolates sequenced from section Nigri comprised *Aspergillus phoenicis* (12/18), *A. niger* (5/18) and *A. tubingensis* (1/18). Other *Aspergillus* isolates identified were *A. versicolour* (4), *A. sclerotiorum* (2) and *A. ochraceus* (1). (See Table 3).

3.4. Dose–response tests

The fungal treatments A. *flavus* (G49), *A. fumigatus* (G23) and *A. niger* (L29) in Experiment 1a differed significantly in their effect on the survival of larvae ($\chi^2_{3,307} = 217.61$, $P < 0.001$), and there was also a significant effect of colony ($\chi^2_{3,300} = 16.25$, $P < 0.001$). The effect of dose differed depending on the species, although this was not significant ($\chi^2_{4,303} = 8.59$, $P = 0.07$). Larvae treated with *A. flavus* (G49) showed significantly higher mortality than the controls in all doses ($P < 0.001$ in all

Table 2
The percentage of cultures in which *Aspergillus* isolates from each group were identified by gross morphology, including the pooled (10 adult bee guts or larvae per colony), individual (20 nurse bee gut, large larvae or small larvae individuals from 3 colonies) and soil cultures (hive entrance and 5 m from 11 hives).

Aspergillus group	Pooled cultures ($n = 39$)		Individual cultures ($n = 60$)			Soil ($n = 11$)	
	Adults	Larvae	Nurse bees	Large larvae	Small larvae	Hive	Grass
Fumigati	60	72	28	68	26	45	27
Nigri	33	31	45	7	7	27	18
Flavi	23	13	3	3	3	0	0

Table 3
The *Aspergillus* isolates sequenced from the between-colony (pooled cultures) and within-colony (individual cultures) and airborne samples and including subset of non-Aspergillus isolates that were also sequenced.

Sample code	Species	Genbank ID
Between colony samples (n = 39)		
Adults		
G5	*A. phoenicis*	KJ123903
G7	*A. nomius*	KJ123914
G15	*A. fumigatus*	KJ123923
G20	*A. fumigatus*	KJ123915
G23	*A. fumigatus*	KJ123924
G30	*A. fumigatus*	KJ123925
G49	*A. flavus*	KJ123911
Larvae		
L27	*A. fumigatus*	KJ123916
L29	*A. niger*	KJ123900
L29b	*A. versicolour*	KJ123926
L38	*A. oryzae*	KJ123917
Within-colony samples (n = 3)		
Nurse bees		
N30.02	*A. sclerotiorum*	KJ123929
N30.04	*A. fumigatus*	KJ123930
N30.04b	*A. niger*	KJ123908
N30.10	*A. sclerotiorum*	KJ123931
N30.17	*A. fumigatus*	KJ123934
N38.01	*A. fumigatus*	KJ123935
N38.02	*A. phoenicis*	KJ123901
N38.09	*A. phoenicis*	KJ123904
N38.11	*A. phoenicis*	KJ123893
N38.14	*A. phoenicis*	KJ123894
N38.16	*A. niger*	KJ123909
N40.02	*A. phoenicis*	KJ123895
N40.03	*A. phoenicis*	KJ123905
N40.04	*A. phoenicis*	KJ123902
N40.10	*A. niger*	KJ123906
N40.11	*A. phoenicis*	KJ123896
N40.15	*A. fumigatus*	KJ123936
N40.15	*A. phoenicis*	KJ123897
N40.16	*A. tubingensis*	KJ123910
N40.17	*A. phoenicis*	KJ123898
N40.18	*A. phoenicis*	KJ123899
Larvae		
LL30.08	*A. fumigatus*	KJ123918
LL30.13	*A. versicolour*	KJ123919
LL30.13	*A. niger*	KJ123907
LL38.03	*A. fumigatus*	KJ123927
LL38.15	*A. fumigatus*	KJ123920
LL40.18	*A. fumigatus*	KJ123928
LS30.02	*A. versicolor*	KJ123932
LS30.02b	*A. flavus*	KJ123933
LS30.08	*A. versicolour*	KJ123921
Airborne		
ABB38	*A. ochraceus*	KJ123922
ABM30	*A. fumigatus*	KJ123913
ABM40	*A. oryzae*	KJ123912
Non-Aspergillus isolates		
G11	*Penicillium Glabrum*	KJ123941
G13	*Purpureocillium lilacinum*	KJ123942
L2	*Fusarium* sp.	KJ123943
L3	*Fusarium oxysporum*	KJ123944
L4	*Penicillium ilerdanum*	KJ123945
L7	*Penicillium crustosum*	KJ123946
L9	*Ascosphaera apis*	KJ123947
L30	*Penicillium ilerdanum*	KJ123948
N38.17	*Penicillium commune*	KJ123949
LL30.11	*Penicillium spinulosum*	KJ123950
4H	*Fusarium tricinctum*	KJ123938
45Hb	*Penicillium tricinctum*	KJ123940
45Hd	*Penicillium crustosum*	KJ123939

*Source code letters: G = adult bee guts, L = larvae, N = nurse bee gut, LL = large larva, LS = small larva, ABB = bottom airborne, ABM = mid airborne, H = hive entrance soil.
Numbers denote the colony or the colony and individual number.

cases; Fig. 1), whereas the survival of larvae was not affected significantly by any of the *A. fumigatus* (G23) ($P > 0.05$ in all cases) and *A. niger* (L29) treatments ($P > 0.05$ in all cases; Fig. 1). In Experiment 1b the effect of the fungal treatments *A. fumigatus* (N30.04) and *A. phoenicis* (N38.02) species on the survival of larvae was significant ($\chi^2_{2,566} = 110.53, P < 0.001$) which was affected by both dose ($\chi^2_{4,562} = 27.24$, $P < 0.001$) and colony ($\chi^2_{3,559} = 54.48$, $P < 0.001$). Higher mortalities were observed in larvae with increasing doses of *A. phoenicis* treatments showing a clear dose response effect (Fig. 1). All doses showed a significantly greater effect on mortality than the control treatments. In Experiment 1c there was a significant effect of parasite species ($\chi^2_{2,567} = 88.13$, $P < 0.001$) and dose ($\chi^2_{4,563} = 24.93$, $P < 0.001$) on the survival of larvae treated with *A. flavus* (G49) and *A. nomius* (G7). There was no significant effect of colony on the survival of larvae overall ($\chi^2_{3,560} = 5.04, P = 0.17$). Again, in all *A. flavus* (G49) treatments the mortality of larvae was significantly greater than the control treatment ($P < 0.01$ in all cases). However, colony was not a significant factor in larval mortalities exposed to *A. flavus* (G49) treatments in this experiment ($\chi^2_{3,370} = 3.92$, $P = 0.27$). There was a significant effect of dose ($\chi^2_{4,373} = 21.09$, $P < 0.001$) and colony ($\chi^2_{3,370} = 34.06, P < 0.001$) on the survival of larvae treated with *A. nomius* (G7) with the two highest doses (50 and 500 conidia) showing significantly higher mortality than the controls.

There was a significant effect of *A. flavus* treatment on the survival of adult bees ($\chi^2_{1,447} = 156.46, P < 0.001$). The overall effect of dose was significant ($\chi^2_{4,443} = 103.23$, $P < 0.001$) with all doses causing significantly higher mortality than the control treatments. There was also a significant frailty effect of unobserved heterogeneity between colonies ($\chi^2_{0.005,443} = 53.23, P < 0.001$, Fig. 2).

4. Discussion

Honey bees are exposed to a high diversity and abundance of fungal conidia in the hive environment. This was evident in the high CFU intensities found in larvae, adult honey bees and airborne samples collected in this study. High prevalences of *Aspergillus* spp. fungi were also observed, and we note that these results are a conservative measure as many non-conidiating colonies were not included and actual prevalences are likely to be higher. Three (*A. flavus*, *A nomius* and *A. phoenicis*) of the ten species identified were pathogenic to honey bee larvae. Moreover, adult honey bees were also confirmed to be highly susceptible to *A. flavus* infection when they ingested conidia.

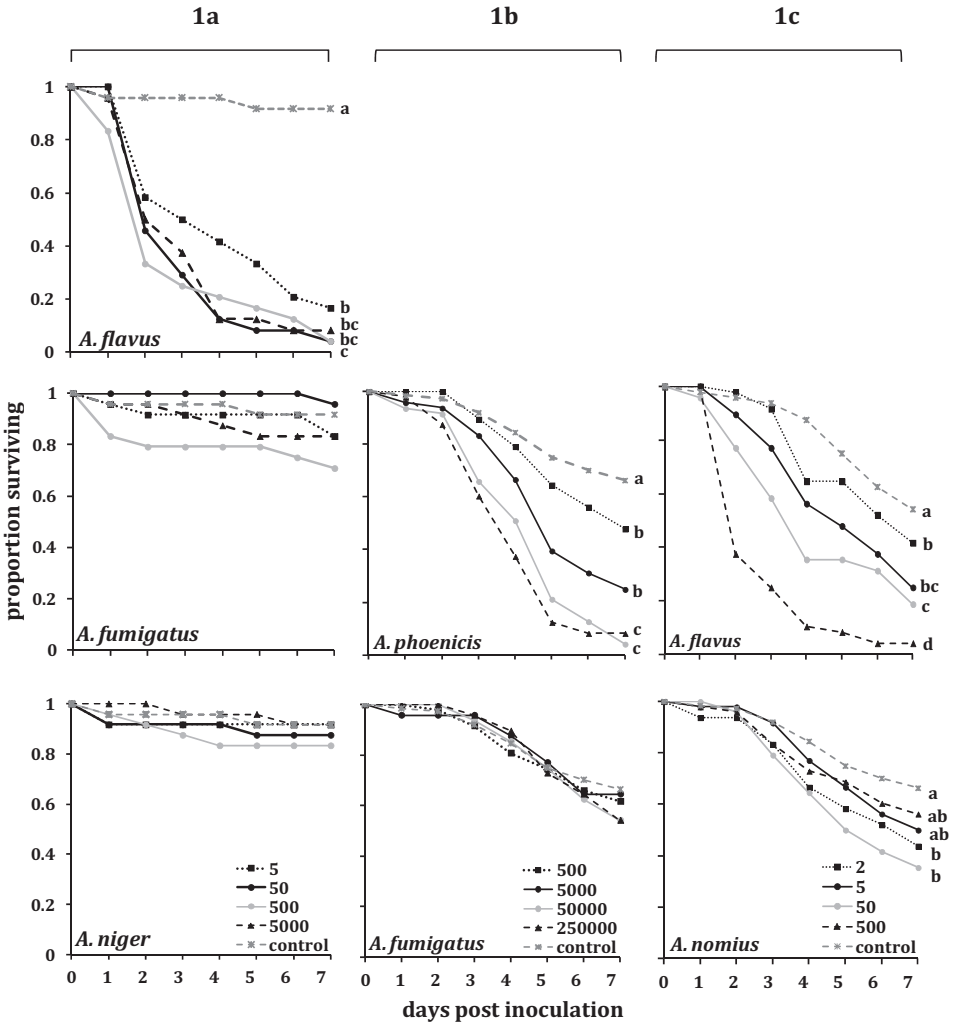

Fig. 1. The survival rates of larvae over 7 days following fungal treatment in Experiment 1a (left column) for: *A. flavus* (G49), *A. fumigatus* (G23) and *A. niger* (L29), Experiment 1b (centre column): *A. phoenicis* (N38.02) and *A. fumigatus* (N30.04) and Experiment 1c (right column): *A. flavus* (G49) and *A. nomius* (G7) with a different dose range in each experiment. For each experiment, the dose legend is shown in the bottom graph. The different letters denote significant differences in pair-wise comparisons between doses for each *Aspergillus* spp. isolate.

The genus *Aspergillus* consists of over 250 species of which 40 are known to be opportunistic pathogens (Varga and Samson, 2008). We found that of the most frequently occurring isolates belonging to section Fumigati, all were confirmed as *A. fumigatus.* Neither of the two *A. fumigatus* isolates used in the dose–response tests induced mortality in larvae and were the least pathogenic of all the fungal isolates tested. The majority of isolates from the next most common section Nigri, were confirmed to be *A. phoenicis*. A dose–response test using an *A. phoenicis* (N38.02) isolate showed increased mortality in all doses and is the first report of this species showing pathogenicity in bees. The *A. niger* (L29) isolate was not pathogenic and illustrates how subtle phenotypic variation between cryptic *Aspergillus* spp. can significantly affect their virulence underscoring the importance of accurate microbial identification in epidemiological studies. The dose–response tests using *A. flavus* and *A. nomius* strains from the least common section Flavi, showed *A. flavus* to be pathogenic at all doses. This high virulence has also been reported by Vojvodic et al. (2011) using an *A. flavus* isolate sourced from honey bee larvae, which showed higher virulence than the obligate brood pathogen *Ascosphaera apis* in larvae reared under the same conditions at the same dose. This is also interesting as *A. flavus* conidiates more prolifically than *A. apis* produces infective ascospores, presumably releasing higher densities of infective propagules into the environment, yet appears to cause disease outbreaks far less frequently than *A. apis*. A clear dose–response with *A. nomius* treatment showed it to be pathogenic at doses of 50 conidia or more. *A. nomius* is commonly isolated from insects and is a known entomopathogen; it is also a producer of A and G-type aflatoxins (Poulsen et al., 2006). The high virulence of *A. flavus* was also evident in the adult bee experiment where all doses were pathogenic. Similarly, high mortalities were reported by Burnside (1930) except using much higher and uncontrolled dosing methods. He also noted that, in free flying colonies,

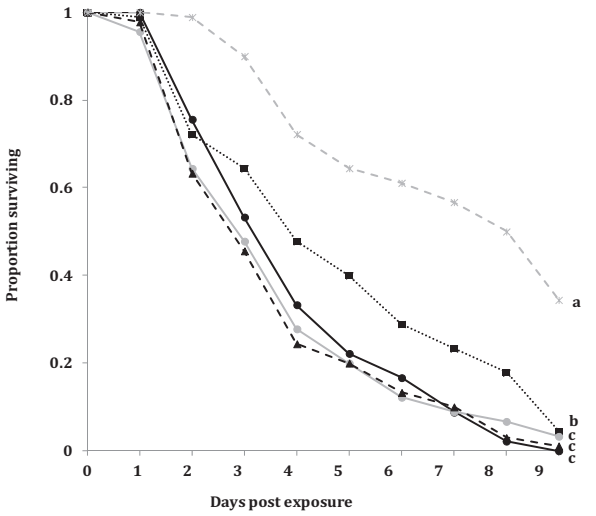

Fig. 2. The survival rates of adult bees over 9 days following *A. flavus* (G49) treatment for each dose of 25,000 conidia (black triangles), 5000 conidia (grey circles), 500 conidia (black circles), 50 conidia (black squares) and controls (grey crosses). The different letters denote significant differences in pair-wise comparisons between doses.

infected bees left the hive to die. These results are noteworthy as the importance of aspergillosis as an adult bee disease may be underestimated in comparison to its importance as a brood disease.

Viable conidia intensities showed highly aggregated distribution which may suggest variation in exposure to fungal conidia. Larvae generally carried higher conidia loads than the adult bee gut samples. This may be partly accounted for by the inclusion of cuticle surface in the larval samples and the retention of conidia in the larval gut until the first defecation event prior to pupation. The larval CFU counts represent the accumulation of conidia ingested during development, reflected in the relatively low CFU counts in individual small larvae. One of the main sources of the fungi in the hive are most likely to be from pollen, as nectar is not thought to harbour large quantities of fungal conidia (González et al., 2005). Conidia present in the air may also colonise hive substrates saprophytically and produce conidia which may become airborne in the hive or transmitted via physical contact or food sharing in adult bees. Conidia may then in turn be fed to larvae in the cells. The *V. destructor* mite may also potentially act as vector of the fungal disease (Benoit et al., 2004). Our results show how the ingestion of conidia and infection route through the gut is successful in both larvae and adults with a number of species of *Aspergillus*. The presence of higher densities of conidia on colony substrates will increase the likelihood of infection. Environmental conditions could potentially facilitate the build-up of *Aspergillus* populations increasing the risk of infection.

Completion of the genome sequencing of nine *Aspergillus* species has facilitated numerous investigations into the genetic basis of pathogenicity characters in the genus (see http://www.aspergillus.org.uk/). *Aspergillus flavus* is the most common *Aspergillus* species reported to infect insects and second most common in human aspergillosis (Hedayati et al., 2007 and St. Leger et al., 1997). Murine studies have shown *A. flavus* doses having 100-fold higher infectivity when compared to *A. fumigatus* (Mosquera et al., 2001). Damage to the host tissues during mycoses can result either from the mechanical action exerted by the mycelium during growth or chemically by enzymes and toxins secreted by the fungus (Rosengaus and Traniello, 1997). The toxicity of aflatoxin to honey bees has been investigated in a few studies and suggests a high tolerance for the aflatoxin B1 compound due to P450-mediated metabolic detoxification (Niu et al., 2011). Propolis extracts and a honey diet also ameliorated toxicity effects in bees (Johnson et al., 2012; Niu et al., 2011). These studies suggest that bees have a capacity to cope with mycotoxins they may be exposed to but the role mycotoxins play in the pathogenicity of *Aspergillus* spp. infections has yet to be examined.

Given the high mortality of larvae and adult bees in this study, it is apparent that individual immune responses are easily overcome by certain *Aspergillus* strains. This suggests that colony level defences play a vital role in the defences against aspergillosis under natural conditions. Hygienic behaviour is a specific response by adult bees to the presence of diseased or parasitised brood and is an important defence against larval diseases such as American foulbrood, chalkbrood and *V. destructor* mite infestations in *Apis ceranae* (Gilliam et al., 1983; Evans and Spivak, 2010). For the behaviour to be effective the bees must detect and remove infected larvae before the infective transmission stage of the disease is reached. It is possible that this is routinely carried out in colonies in response to stonebrood and has gone undetected. The removal of larvae that were experimentally administered high doses of *A. flavus* conidia has been observed previously, however this mechanism still requires further investigation under controlled conditions to determine if it is characteristic of hygienic behaviour (Burnside, 1930). Nest hygiene defined as behaviours that increase sanitation of an individual or the nest (Wilson-Rich et al., 2009) is another possible defence mechanism of high importance against stonebrood. Larvae are fed royal jelly and stored honey which both possess antimicrobial properties (Morse and Flottum, 1997). The efficacy of propolis as an antimicrobial against honey bee disease is evident with American foulbrood agent *Paenibacillus larvae* where studies have also shown that the antibacterial activity varies depending on the source of the plant resin (Bastos et al., 2008). The presence of propolis on all hive surfaces may act as a persistent barrier against the establishment of harmful fungi. The presence of plant resins in the nest is known to result in the down-regulation of immune gene expression in individual bees emphasising a significant role for propolis in disease resistance and colony fitness (Simone et al., 2009). Bees have been shown to significantly increase their resin foraging rate in response to chalkbrood challenge, more so than to American foulbrood, indicating a specific response to this long-lasting fungal pathogen (Simone-Finstrom and Spivak, 2010).

In conclusion, our results indicate that *Aspergillus* fungi are ubiquitous in the environment of honey bees and may pose a significant stress on the health of their colonies. Under experimental conditions both adults and larvae from apparently healthy colonies, were found to be

susceptible to infection by *Aspergillus* fungi isolated from honey bees. It is clear that further research is required to determine how colonies coexist with these potentially harmful organisms and to identify the key defences involved in aspergillosis prevention.

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